Environmental Health Perspectives
Vol. 19, pp. 209–213, 1977

Effects of Chronic Arsenic Exposure on Hematopoietic Function in Adult Mammalian Liver

by James S. Woods* and Bruce A. Fowler*

In these studies the effects of ingested arsenic (As^{+5}) on hepatic heme biosynthetic capability and hemoprotein function in adult male rats were investigated. Animals exposed for 6 weeks to 0, 20, 40, or 85 ppm sodium arsenate in the drinking water suffered depression of hepatic δ-aminolevulinic acid (ALA) synthetase and heme synthetase (ferrochelatase) activities, with maximal decreases to 67 and 55% of control levels, respectively, at 85 ppm. Concomitantly, urinary uroporphyrin levels were elevated by as much as 12 times, and coproporphyrin by as much as 9 times, control values. The rate of incorporation of ^{3}H-ALA into mitochondrial and microsomal hemes was depressed by 40–50% at 20 ppm but was increased with regard to controls by as much as 150% at the higher treatment levels. A similar biphasic pattern was observed in regard to ^{14}C-leucine incorporation into cellular membranal proteins. In contrast, the levels of ALA dehydratase, uroporphyrinogen I synthetase, aminopyrine demethylase, and cytochrome P-450 were not significantly changed in As^{+5}-treated rats. These results support the hypothesis that chronic, low level, arsenic exposure results in selective inhibition of mitochondrial-bound heme biosynthetic pathway enzymes (ALA synthetase and heme synthetase) resulting in a substantial increase in urinary porphyrins, uniquely characterized by a greater increase in uroporphyrin than coproporphyrin levels. These changes occur independent of, or prior to, alterations in hepatic hemoprotein-dependent functions and may thus serve in the clinical analysis of pretoxic exposure to arsenic compounds in human populations.

Introduction

Previous papers in this session have attested to the variety of potentially toxic effects of arsenic compounds on specific organ functions following chronic low-level exposure in mammals. In this paper the results of experiments which were designed to assess the effects of inorganic arsenic compounds on hematopoietic function in adult mammalian liver are described.

Figure 1 presents a schematic representation of the heme biosynthetic pathway, which is responsible for hematopoiesis in adult mammalian tissues. This process in normal tissues is regulated primarily by the first and rate-limiting enzyme in this pathway, δ-aminolevulinic acid (ALA) synthetase (*1*). Other important enzymes in the heme biosynthetic pathway include ALA dehydratase, uroporphyrinogen I synthetase, and heme synthetase (ferrochelatase), all of which are known to limit heme biosynthetic capability under various physiological or toxicological conditions (*2*).

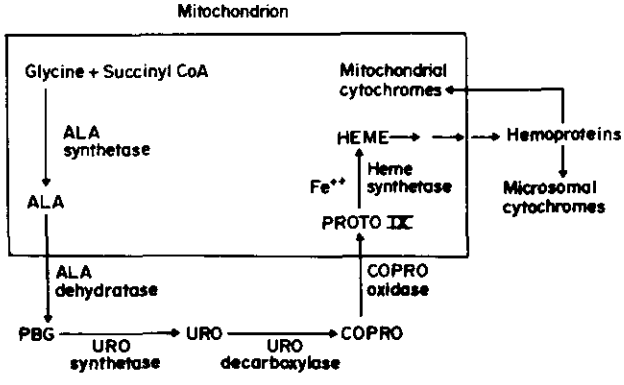

FIGURE 1. Heme biosynthetic pathway: URO=uroporphyrinogen, COPRO=coproporphyrinogen, PROTO=protoporphyrin.

*Environmental Toxicology Branch, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, North Carolina 27709.

The hematopoietic system in organs such as liver, kidney, and other nonerythropoietic tissues plays the extremely important role in these organs of providing heme for the biosynthesis of hemoproteins such as the mitochondrial and microsomal cytochromes, which are essential to cellular growth and function. This system is known to be highly susceptible to alteration by many drugs and other environmental chemicals, particularly trace metals (*2, 3*), and it is altogether appropriate, therefore, that it be considered as a potential target system for arsenic compounds in animals which are chronically exposed to these agents.

In the present studies the potential hematotoxicity of sodium arsenate on hematopoietic function in adult rat liver was investigated. A variety of parameters designed to measure the effects of arsenic on both heme biosynthetic capability and hemoprotein function were examined, and the utility of the findings in the prediction of pretoxic exposure to environmental arsenic compounds in human populations was assessed.

Materials and Methods

Treatment of Animals

Male Sprague-Dawley rats (CD strain), obtained from the Charles River Laboratories, Boston, Massachusetts, were divided into four groups of 12 animals each and were placed in individual wire bottom cages in barrier isolation rooms. Animals were given access to laboratory chow and deionized drinking water containing 0, 20, 40, or 85 ppm arsenic as sodium arsenate for up to 6 weeks. In urinary porphyrin studies, animals were placed in individual metabolism cages for the collection of urine over a 16-hr period immediately preceding the analysis of urinary heme precursors. In pulse-labeling studies, animals were given 20 μCi ^{14}C-leucine or 60 μCi δ-aminolevulinic-3,5-^{3}H(N) acid by intraperitoneal injection 10 min prior to sacrifice. Isotopes were obtained from New England Nuclear, Boston, Massachusetts.

Preparation of Tissues

Animals were killed by decapitation. Livers were rapidly excised, washed, weighed, and homogenized in 9 volumes of 0.25*M* sucrose containing 0.05*M* Tris-HCl buffer, pH 7.5 in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Mitochondria and microsomal fractions were then prepared as previously described (*4*).

Assay of Enzyme Activities

ALA synthetase activity in hepatic mitochondria was measured as previously described (*4*). Reaction mixtures contained approximately 4 mg mitochondrial protein/ml.

ALA dehydratase activity was measured in 9000 *g* supernatant fractions of liver and kidney homogenates by a modification of the method of Gibson et al. (*5*), as described by Baron and Tephly (*6*).

Uroporphyrinogen I synthetase activity in liver and kidney preparations was measured in the 9000 *g* supernatant fractions after heating at 65°C for 15 min, as previously described (*2*).

Mitochondrial ferrochelatase activity was measured by a modification of the method of Porra (*7*) by using mesoporphyrin-IX dihydrochloride as substrate. Reaction mixtures containing 0.25 μmole mesoporphyrin-IX, 4.0 μmole reduced glutathione, 0.5 μmole $FeSO_4$, 1.65 ml of 0.05*M* Tris-HCl buffer, pH 7.6, and 10 mg of mitochondrial protein in a total volume of 3.25 ml were flushed extensively with oxygen-free N_2, capped with anerobic test-tube stoppers, and incubated with shaking for 1 hr at 37°C in a N_2 atmosphere. Reactions were terminated by the addition of 0.3 ml of 0.4*M* iodoacetamide, 1 ml pyridine, 0.5 ml 1*N* NaOH and 2.5 ml water. Mixtures were then centrifuged for 10 min at 9500 rpm. The clear supernatant solution was divided between two matched cuvettes, and the reduced minus oxidized spectrum, obtained after adding $Na_2S_2O_4$ or $K_3Fe(CN)_6$ to separate cuvettes, was recorded from 600 to 500 nm by using a Beckman Acta III recording spectrophotometer. The concentrations of the pyridine mesohemochromes were calculated as described by Porra (*7*), a value of 21.7 being used for the molar absorbance.

Cytochrome P-450 levels in rat liver microsomes were determined by difference spectrophotometry using the method of Omura and Sato (*8*). Microsomal aminopyrine demethylase and mitochondrial cytochrome oxidase activities were measured as previously described (*2*).

Protein determinations were made by the method of Lowry et al. (*9*) using bovine serum albumin (Fraction V) as a standard.

Quantitation and Assessment of ^{3}H-ALA Incorporation Into Mitochondrial Hemes

Mitochondrial hemes were extracted according to the method of Rieske (*10*). The mitochondrial pellet was homogenized successively with acetone, chloroform:methanol (2:1, v/v), and acetone. The supernatant solution remaining after centrifugation at 9000 rpm for 15 min was discarded, and the pellet was extracted 3 times for 15 min each with 10 volumes of acetone:HCl (0.9 ml of concentrated HCl per 100 ml of acetone). The combined acetone:HCl extracts were then evaporated to dryness in vacuo.

The residue was immediately dissolved in 1 ml of pyridine, and an aliquot was removed for counting in a Packard Tri-Carb liquid scintillation spectrometer with appropriate corrections for quenching. Measurements were made in glass counting vials using 10 ml Biofluor (New England Nuclear) as counting solution. The heme content was determined by the pyridine hemochromogen method as described by Rieske (*10*).

Quantitation and Assessment of ^{3}H-ALA Incorporation Into Microsomal Hemes

Microsomal hemes were quantitated by the pyridine hemochromogen method as described by Falk (*11*). Microsomal pellets were suspended in 0.05*M* Tris-HCl buffer, pH 7.5, so as to contain approximately 2 to 3 mg of protein/ml. An aliquot was removed for counting in a liquid scintillation spectrometer by using 20 ml of PPO–POPOP counting solution. The microsomal heme content was calculated from the difference in absorption between 541 and 557 nm, a value of 20.7 being used for the molar absorbance.

Assessment of ^{14}C-Leucine Incorporation into Mitochondrial and Microsomal Fractions

In this step, 1-ml fractions of washed mitochondrial or microsomal pellets were dissolved in equal portions of 0.4*N* NaOH, and aliquots were counted in a liquid scintillation spectrometer, as described above.

Quantitation of Urinary Heme Precursors

Urinary ALA and PBG were determined by the colorimetric procedure described by Mauzerall and Granick (*12*) and modified by Davis and Andelman (*13*). Urinary levels of uroporphyrin and coproporphyrin were determined fluorometrically, as described by Talman and Schwartz (*14*).

Results and Discussion

The effects of continuous exposure to sodium arsenate on the activities of heme biosynthetic pathway enzymes in rat liver are presented in Figure 2. Exposure to arsenic at the lowest dose level (20 ppm) had very little effect on any of these enzymes. The one significant change which occurred at this dose level was an approximate 20% inhibition of heme synthetase, the final enzyme in the heme biosynthetic pathway. With increasing dose levels of arsenic exposure, heme synthetase was further depressed, achieving a maximal decrease in activity to approximately 55% of control (0 ppm) levels at 85 ppm. Concomitantly, ALA synthetase was also depressed at higher arsenic dose levels, attaining a maximal depression to approximately 67% of control levels at 85 ppm.

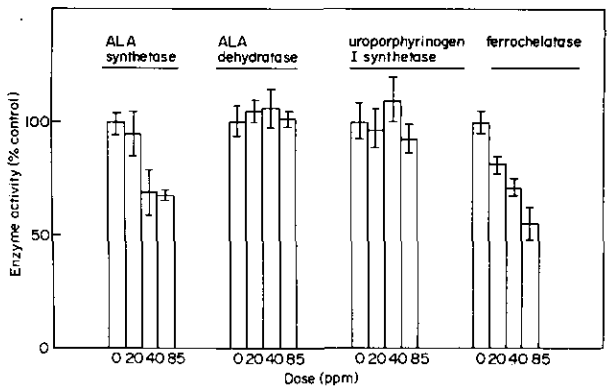

FIGURE 2. Effects of exposure to sodium arsenate on activities of heme biosynthetic pathway enzymes in rat liver. Values in this and subsequent figures represent means ± standard errors of at least five experiments and are expressed as percent of control.

Accompanying this decrease in heme biosynthetic pathway enzyme activities was a substantial elevation of urinary uroporphyrin and coproporphyrin levels in animals exposed to high doses of arsenic. As indicated in Figure 3, uroporphyrin levels were increased to almost 12 times control values, whereas coproporphyrin was increased to approximately 9 times the controls. This observation is of particular interest, since in most known cases of chemical prophyria coproporphyrin levels almost always exceed those of uroporphyrin (*15*). This unusual pattern of urinary porphyrin excretion, therefore, may be uniquely characteristic of arsenic exposure, an observation which could be of utility in the detection of subclinical arsenic toxicity in humans.

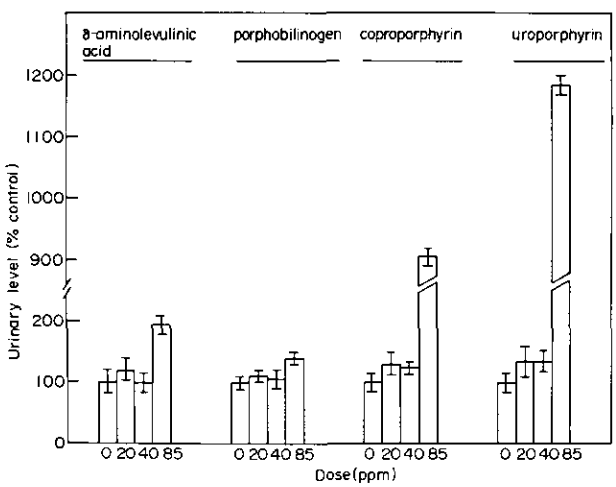

FIGURE 3. Levels of porphyrins and heme precursors in urine of rats during chronic exposure to sodium arsenate.

The observed changes in heme biosynthetic pathway enzymes and urinary prophyrin levels occurred independently of, or prior to, any overall changes in hemoprotein function in the liver (Fig. 4). Levels of aminopyrine demethylase and cytochrome P-450, which serve as indicators of microsomal hemoprotein function, were not substantially altered in arsenic-treated animals. Levels of cytochrome oxidase, a measure of mitochondrial hemoprotein function, were actually increased in livers of arsenic-exposed rats, although no change from the control levels in the activity of this enzyme were seen in arsenic-treated mouse liver (unpublished observations). Thus, elevation of urinary porphyrin levels occurs prior to the onset of overt changes in hepatic hemoprotein function and may represent one of the initial manifestations of arsenic exposure.

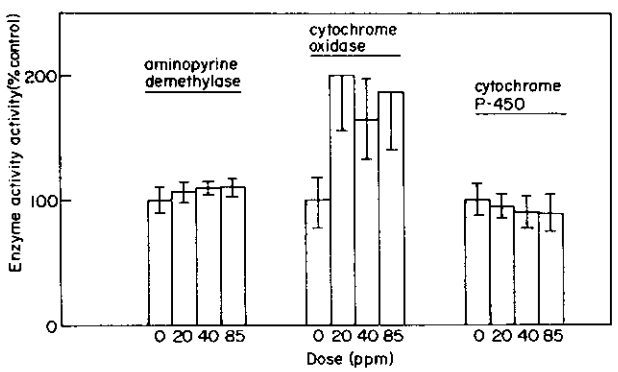

FIGURE 4. Effects of exposure to sodium arsenate on cytochrome oxidase and mixed function oxidase activities in rat liver.

Perhaps a more sensitive indicator of heme biosynthetic capability in mammalian tissues is measured by the rate of incorporation of radiolabeled heme precursors into mitochondrial and microsomal hemes of exposed animals. It was, therefore, of interest to determine if differences in heme biosynthetic capability, as measured by the pulse labeling technique, in control versus arsenic-exposed rats could be observed. In these studies, animals were given pulse doses of tritiated ALA (60 μCi), and the rate of incorporation of the labeled heme precursor into hepatic mitochondrial and microsomal hemes *in vivo* was measured per unit of time. The results of these experiments are shown in Figure 5. These experiments indicated that the rate of incorporation of H-3-ALA into both mitochondrial and microsomal hemes was substantially reduced by as much as 40–50% at the 20 ppm exposure level. At higher levels, however, a paradoxical reversal of this reduced incorporation phenomenon was observed, with the rate of incorporation of ^{3}H-ALA into hepatic hemes reaching almost 200% with regard to controls at 85 ppm.

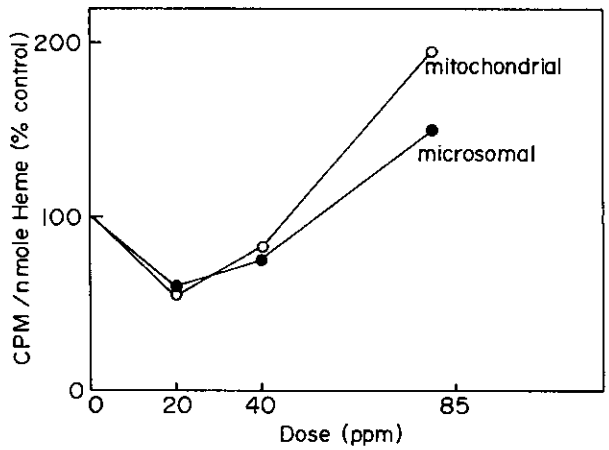

FIGURE 5. Incorporation of ^{3}H-ALA into hepatic mitochondrial and microsomal hemes during chronic exposure to sodium arsenate in rats.

This observation suggested an actual increase in the utilization of hepatic heme at higher arsenic exposure levels. Since the specific activities of hemoproteins were not increased in livers of arsenic-treated rats, however, and since the levels of heme biosynthetic pathway enzymes were actually decreased in these animals, it did not appear that these incorporation patterns could be attributed to specific changes in heme biosynthetic capability or an increased demand for heme per se. Therefore, further pulse-labeling studies were performed in order to determine if the increase in labeling of hepatic hemes following arsenic exposure was a reflection of a more general alteration in hepatocellular proliferative activity. In these studies, animals were pulse labeled with ^{14}C-leucine (20 μCi), and the rate of incorporation of this precursor into the membranal proteins of cellular organelles was examined.

Again, a biphasic pattern of incorporation, similar to that seen in the case of ^{3}H-ALA was observed (Fig. 6). A marked depression of the incorporation of leucine into cellular proteins which occurred after exposure to low levels of arsenic was translated into an actual stimulation of this activity at higher levels of exposure.

These studies suggest that the increased labeling of hepatic hemes observed in arsenic-exposed rats is not a specific effect but rather is probably a reflection of a more general proliferation of cellular membranal components which occurs during arsenic exposure. The rapid labeling of both hemes and membranal proteins in liver cells of arsenic exposed rats provides a biochemical correlation with the ultrastructural studies previously described (*16*), demonstrating the rapid proliferation of cellular membranal components in arsenic-treated animals. This type of observation, in which one finds a hyper-

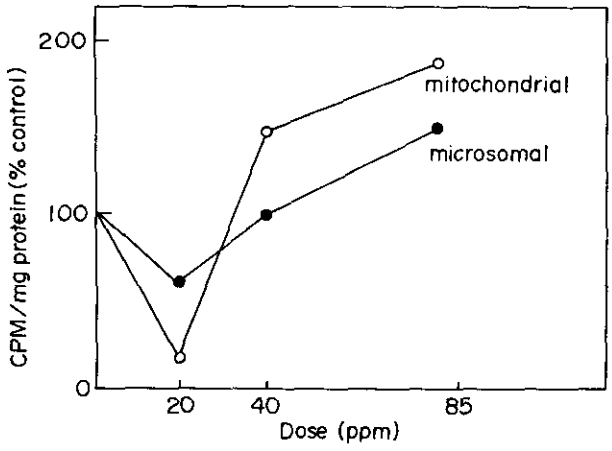

FIGURE 6. Incorporation of ^{14}C-leucine into hepatic mitochondrial and microsomal proteins during chronic exposure to sodium arsenate in rats.

trophic proliferation of membranes of cellular organelles, which is not associated with a corresponding increase in the activities of many of the enzymes which are normally found in these membrane systems, is one which is characteristic of the response of the liver to a variety of chemicals during the induction of chronic liver disease (*17*).

While no specific manifestations of chronic liver disease nor loss or organ function have been demonstrated in these investigations, these studies do suggest that such consequences could result after chronic exposure to arsenical agents. It is, therefore, of the greatest interest that the identification of biochemical and other metabolic changes which occur during the early phases of arsenic exposure be made. Among these alterations identified in the present studies are specific changes in hepatic heme biosynthetic pathway enzymes, resulting in an unusual pattern of porphyrin excretion in urine of arsenic exposed animals. This pattern differs from those produced by most other prophyrogenic environmental agents (*3*, *15*) and may, therefore, be of value in the design of clinically useful tests for monitoring pretoxic exposure to arsenic compounds in human populations.

REFERENCES

1. Granick, S., and Urata, G. Increase in the activity of δ-aminolevulinic acid synthetase in liver mitochondria induced by feeding 3,5-dicarboxyethyl-1, 4-dihydrocollidine. J. Biol. Chem. 238: 821 (1963).
2. Woods, J. S. Developmental aspects of hepatic heme biosynthetic capability and hematotoxicity. Biochem. Pharmacol. 25: 2147 (1976).
3. Woods, J. S., and Fowler, B. A. Renal porphyrinuria during chronic methyl mercury exposure. J. Lab. Clin. Med., in press.
4. Woods, J. S. Studies on the role of heme on the regulation of δ-aminolevulinic acid synthetase during fetal hepatic development. Molec. Pharmacol. 10: 389 (1974).
5. Gipson, K. D., Neuberger, A., and Scott, J. J. The purification and properties of δ-aminolevulinic acid dehydrase. Biochem. J. 61: 618 (1955).
6. Baron, J., and Tephly, T. R. Effect of 3-amino-1,2,4-triazole on the induction of rat hepatic microsomal oxidases, cytochrome P-450, and NADPH-cytochrome reductase by phenobarbital. Mol. Pharmacol. 5: 10 (1969).
7. Porra, R. J. A rapid spectrophotometric assay for ferrochelatase activity in preparations containing much endogenous hemoglobin and its application to soybean root-nodule preparations. Anal. Biochem. 68: 289 (1975).
8. Omura, T., and Sato, R. The carbon monoxide binding pigment of liver microsomes. I. Evidence for hemoprotein nature. J. Biol. Chem. 239: 2370 (1964).
9. Lowry, O. H., et al. Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265 (1951).
10. Rieske, J. S. The quantitative determination of mitochondrial hemoproteins Methods Enzymol. 10: 488 (1967).
11. Falk, J. E. In Porphyrins and Metalloporphyrins, Elsevier, Amsterdam, 1964, p. 182.
12. Mauzerall, D. and Granick, S. The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. J. Biol. Chem. 219: 435 (1956).
13. Davis, J. R., and Andelman, S. L. Urinary delta-aminolevulinic acid (ALA) levels in lead poisoning. I. A modified method for the rapid determination of urinary delta-aminolevulinic acid using disposable ion-exchange chromatography columns. Arch. Environ. Health 15: 53 (1967).
14. Talman, E. L., and Schwartz, S. Porphyrins in urine. In: Standard Methods in Clinical Chemistry, II, 1957, pp. 137.
15. Tschudy, D. P., and Bonkowsky, H. L. Experimental porphyria. Fed. Proc. 31: 147 (1972).
16. Fowler, B. A., Woods, J. S., and Schiller, C. M. The ultrastructural and biochemical effects of oral arsenic exposure in liver mitochondria of rats. Environ. Health Perspect. 19: 00 (1977).
17. Gravela, E., et al. Functional and structural alterations of liver ergastoplasmic membranes during DL-ethionine hepatocarcinogenesis. Cancer Res. 35: 3041 (1975).